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Evaluation of the Mutagenicity and Carcinogenicity of Motor Vehicle Emissions in Short-Term Bioassays

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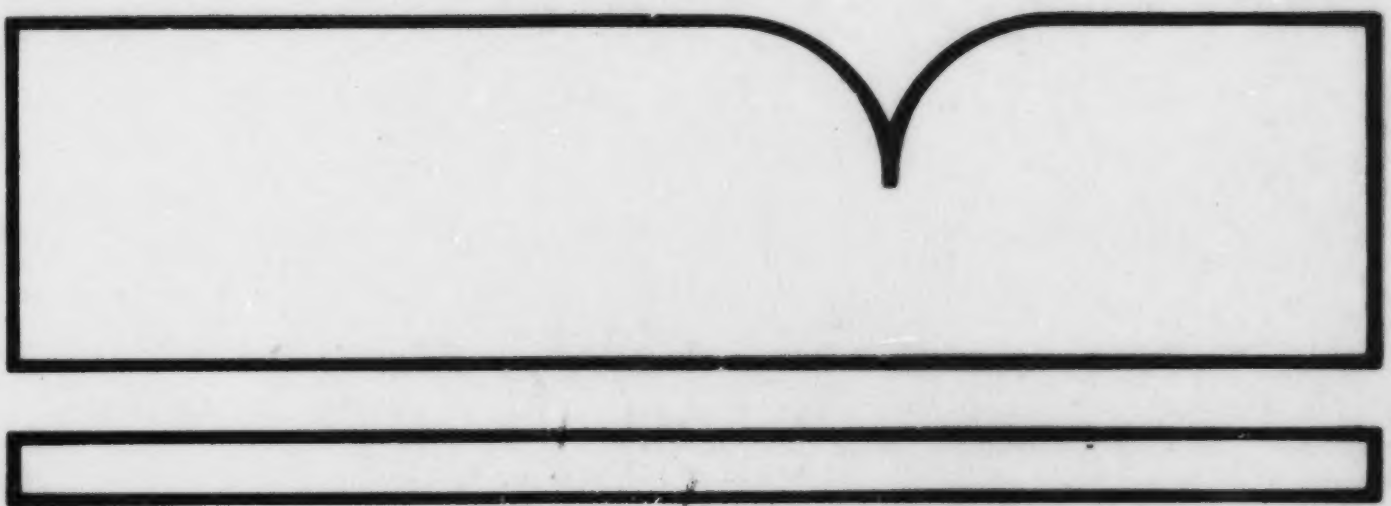

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16. ABSTRACT

Incomplete combustion of fuel in motor vehicles results in the emission of submicron carbonaceous particles which, after cooling and dilution, contain varying quantities of extractable organic constituents. These organics are mutagenic in bacteria. Confirmatory bioassays in mammalian cells provide the capability of detecting chromosomal and DNA damage in addition to gene mutations. In order to evaluate the mutagenicity of these organics in mammalian cells, extractable organics from particle emissions from several diesel and gasoline vehicles were compared in a battery of microbial, mammalian cell and in vivo bioassays. The mammalian cell mutagenicity bioassays were selected to detect gene mutations, DNA damage, and chromosomal effects. Carcinogenesis bioassays conducted included short-term assays for oncogenic transformation and skin tumorigenesis. The results in different assay systems are compared both qualitatively and quantitatively. Good quantitative correlations were observed between several mutagenesis and carcinogenesis bioassays for this series of diesel and gasoline emissions.

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Evaluation of the Mutagenicity and Carcinogenicity of Motor Vehicle Emissions in Short-Term Bioassays

by Joellen Lewtas*

Incomplete combustion of fuel in motor vehicles results in the emission of submicron carbonaceous particles which, after cooling and dilution, contain varying quantities of extractable organic constituents. These organics are mutagenic in bacteria. Confirmatory bioassays in mammalian cells provide the capability of detecting chromosomal and DNA damage in addition to gene mutations. In order to evaluate the mutagenicity of these organics in mammalian cells, extractable organics from particle emissions from several diesel and gasoline vehicles were compared in a battery of microbial, mammalian cell and *in vivo* bioassays. The mammalian cell mutagenicity bioassays were selected to detect gene mutations, DNA damage, and chromosomal effects. Carcinogenesis bioassays conducted included short-term assays for oncogenic transformation and skin tumorigenesis. The results in different assay systems are compared both qualitatively and quantitatively. Good quantitative correlations were observed between several mutagenesis and carcinogenesis bioassays for this series of diesel and gasoline emissions.

Introduction

Short-term microbial mutagenicity bioassays have been extremely useful in evaluating the comparative mutagenicity of different motor vehicle emissions as well as identifying potentially carcinogenic compounds in these complex mixtures (*1-4*). Such studies have led to the identification of nitrated polynuclear aromatic (NO_2-PNA) compounds in diesel emissions (*5,6*). Due to the unusually potent mutagenicity of several of these NO_2-PNAs in bacteria (*7,8*), they may account for much of the observed bacterial mutagenicity.

Concern that bacterial mutagenesis assays may "overestimate" the mutagenic activity of NO_2-PNAs (*9*) in diesel emissions points to the importance of evaluating the mutagenic activity of these and other motor vehicle emissions in eucaryotic organisms, mammalian cells, and whole animals.

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Mammalian cell mutagenesis bioassays capable of detecting gene mutations, DNA damage, and chromosomal aberrations have confirmed the mutagenic activity of diesel emissions (*10,11*). Many of these assays performed with mammalian cell lines, e.g., L5178Y mouse lymphoma cells, BALB/c 3T3 cells, and Chinese hamster ovary (CHO) cells, require the addition of a metabolic activation system containing microsomal as well as other mammalian liver enzymes to metabolize polynuclear aromatic hydrocarbons (PAHs).

The objective of this paper is to review the mutagenic activity of motor vehicle emissions. The organics extractable from emission particles, which may constitute 5 to 50% of the mass of these submicron particles, have been most extensively examined in microbial and mammalian cell mutation assays. This paper compares the microbial mutagenicity, mammalian cell mutagenicity and mouse skin tumorigenicity of these organics. Whole diesel particles, gaseous emissions, and whole exhaust emissions, examined in several different *in vivo* bioassays for both somatic and heritable mutagenic activity, will also be reviewed.

Methodology

Motor Vehicle Emission Samples

The diesel and gasoline particle emissions used in the microbial and mammalian cell mutagenesis studies reported here were collected using dilution tunnel sampling techniques (*11*). The total exhaust from passenger cars or a portion of the exhaust from heavy-duty engines was diluted with filtered air (10:1) prior to collection on 20 in. × 20 in. Teflon-coated Pallflex T60-A20 filters. The mobile sources, fuels, and test conditions are shown in Table 1. The diesel samples were all obtained from vehicles and engines operated on the same lot of No. 2 diesel fuel. The vehicles were operated on a chassis dynamometer, using the highway fuel economy test cycle (HWFET) that averages 48 mph in 12.75 min over 10.24 miles. The engines were operated on engine dynamometers at steady-state operation.

The particle samples collected on 12 to 16 Teflon-coated filters were Soxhlet-extracted with dichloromethane (DCM) in a 2.3 L side-chamber extractor for 48 hr. The Soxhlet-extracted organics were filtered by using Teflon Millipore filters (0.2 μm pore) to remove any remaining particles and concentrated by rotary evaporation under reduced pressure. Aliquots were evaporated to dryness under nitrogen and stored frozen in the dark. The samples were dissolved in dimethyl sulfoxide (DMSO) for all of the bioassays except mouse skin tumorigenesis, oncogenic transformation, and mutation in BALB cells where acetone was used as a solvent.

The whole exhaust emissions employed in the *in vivo* mutagenesis bioassays are described in the references cited for each of those assays.

Bioassays

The mutagenesis bioassays applied to diesel emissions generally included assays for which standard protocols have been developed and validated with a series of individual chemicals. The mutagenesis assays were selected to detect gene mutations, DNA damage, and chromosomal aberrations, as outlined in Table 2. The bioassays were conducted with coded samples at five to seven doses or concentrations after a preliminary toxicity range-finding test. For those assays where a positive dose response was obtained, the slope of the dose-response curve was determined by the linear regression analysis, except for the *S. typhimurium* plate incorporation assay and skin tumor initiation assay where the nonlinear model slope (*12,13*) is compared to the linear regression analysis.

Gene Mutation Assays

Salmonella typhimurium Bioassay

The Ames *S. typhimurium* assay measures histidine reversion in a series of tester strains. The *S. typhimurium* plate incorporation test was conducted as described by Ames et al. (*14*), with minor modifications as described by Claxton (*15*). The modifications included adding the minimal histidine to the plate media rather than to the overlay and incubating for 72 hr rather than 48 hr. Claxton initially reported the specific activity of five of these mobile source samples calculated from linear regression analysis at 100 μg of sample (*15*). These data and those from the additional samples have been reanalyzed using the nonlinear model slope analyses (*12,13*), and are compared to the initial linear slopes as shown in Table 3.

The extractable organics from the diesel particle samples were all mutagenic without the addition of metabolic activation. Comparison of the mutagenic activity in all five tester strains (*16*) showed the diesel samples to be positive in TA 1538, TA 1537, TA 98, and TA 100, and negative in TA 1535. The Caterpillar (Cat) and Volkswagen (VW) Rabbit sam-

Table 1. Mobile source samples.

		Vehicle description	Fuel	Driving cycle	Particle emission rate, g/mi	Extractables, %[c]
Diesel	Cat	Caterpillar 3304	Diesel No. 2	Mode II[a]	—	27.0
	Nissan	Nissan Datsun 220C	Diesel No. 2	HWFET[b]	0.33	8.0
	Olds	Oldsmobile 350	Diesel No. 2	HWFET	0.52	17.0
	VW Rabbit	Volkswagon turbo-charged Rabbit	Diesel No. 2	HWFET	0.18	18.0
Gasoline	Mustang	1977 Mustang II-302, V-8 Catalyst and EGR	Unleaded gasoline	HWFET	0.0053	43.0
	Ford Van	6 cylinder, in line van	Leaded gasoline	HWFET	0.048	19.0

[a]Mode II = 2200 rpm steady state, 85 lb. load.
[b]HWFET = Highway fuel economy cycle 10.24 mi, avg. 48 mph, 12.75 min.
[c] = percent of dichloromethane extractable organics.

Table 2. Outline of the bioassays used to examine the extractable organics motor vehicle particle emissions.

Mutagenesis bioassays
1. Gene mutation assays
   - A. Bacterial
     1. *Salmonella typhimurium*
     2. *Escherichia coli*
   - B. Mammalian cell
     1. Mouse lymphoma, L5178Y
     2. Mouse embryo fibroblasts, BALB/c 3T3
     3. Chinese hamster ovary, CHO
2. DNA damage assays
   - A. Yeast
     1. *Saccharomyces cerevisiae* D3 recombinogenic assay
   - B. Mammalian cell
     1. DNA strand breaks in SHE cells
     2. Unscheduled DNA repair in liver cells
     3. Sister chromatid exchanges in CHO cells
3. Chromosomal aberrations (mammalian cells)
   - A. CHO cells
   - B. Human lymphocytes

Carcinogenesis bioassays
1. Ongogenic transformation assays
   - A. Chemical transformation
     1. Mouse embryo fibroblasts, BALB/c 3T3
     2. Syrian hamster embryo, SHE
   - B. Viral enhancement of transformation
     1. SA7 virus enhancement in SHE cells
2. Skin tumor initiation

ples show increased mutagenic activity in the presence of the S9 activation system, whereas the Nissan and Oldsmobile (Olds) samples show decreased activity in S9 activation. The gasoline particle samples were all less mutagenic in the presence of S9 activation.

A significant difference was observed between the particle emission rates (g/mi) and the percent organic extractable matter for the different vehicles (*12*). The diesel cars emitted approximately 100 times more particles per mile than the unleaded gasoline car. A direct comparison of the mutagenic emission rate for the cars is best expressed as revertants/mile. Claxton and Kohan (*17*) have reviewed the mutagenic emission rates for a number of certification vehicles and found that the diesel vehicles emitted 45 to 800 times as much mutagenic activity per mile as the gasoline catalyst vehicles.

Escherichia coli WP2 Bioassay

The *E. coli* WP2 tryptophan reversion assay is very similar to the *S. typhimurium* plate incorporation assay (*14*) using McCalla's *E. coli* WP2 tryptophan auxotroph (*trp*) with a DNA repair deficiency mutation (uvrA) (*18*). Mortelsmans (*19*) found that the Mercedes diesel sample elicited a reproducible dose-related increase in the number of tryptophan-independent revertants in the absence of metabolic activation. In the presence of metabolic activation, the Mercedes diesel sample was nonmutagenic.

L5178Y Mouse Lymphoma Mutagenesis Assay

The L5178Y mouse lymphoma assay of Clive and Spector (*20*) measures forward mutation frequency at the thymidine kinase (TK) locus. The mouse lymphoma assay was conducted according to the method of Clive et al. (*21*) by Mitchell et al. (*22*) and Cifone and Brusick (*23*) in the evaluation of the mutagenicity of a series of diesel and related environmental emissions. Preliminary dose-range toxicity assays were conducted to select 10 concentrations of each sample that resulted in cell survival of 5 to 90% of the controls. In the mutagenesis assays, duplicate samples were used for each concentration tested. In each assay, 6×10^6 L5178Y $TK^{+/-}$ cells were treated with the organic extracts in 10 mL for 4 hr while rotating in a roller drum at 37°C.

The mutation frequency was calculated by dividing the number of mutant cells per milliliter by the number of viable cells per milliliter at each concentration. Concentrations resulting in less than 10% total relative growth were not used in determining the slope of the mutation response curve for each emission sample, as shown in Table 4.

Table 3. Reverse mutation in *Salmonella typhimurium*.

Fuel	Vehicle description	Model slope; revertants/μg[a] in TA 98		Linear slope; revertants/μg[a] in TA 98			
		−MA	+MA	−MA	(r^2)	+MA	(r^2)
Diesel	Cat	0.32 (0.072–1.4)	1.6 (0.91–2.6)	0.38	(0.74)	0.31	(0.95)
	Nissan	20.8 (19.4–22.2)	15.1 (14.4–15.8)	11.3	(0.98)	13.3	(0.99)
	Olds	2.1 (1.9–2.4)	1.4 (1.2–1.7)	2.2	(0.98)	1.5	(0.96)
	VW Rabbit	5.2 (4.7–5.7)	6.1 (5.3–6.9)	3.8	(0.99)	3.0	(0.99)
Gasoline, unleaded	Mustang	2.1 (1.5–2.9)	8.6 (6.8–10.9)	1.6	(0.97)	3.5	(0.99)
Gasoline, leaded	Ford Van	16.9 (14.1–20.3)	27.1 (23.8–30.8)	11.8	(0.98)	12.8	(0.99)

[a]Nonlinear model slope, revertants/μg, 95% confidence limits shown in parentheses.
[b]Linear regression of the initial slope.

Table 4. Gene mutation in mouse lymphoma L5178Y Cells.[a]

Fuel	Vehicle description	Slope: Mutation frequency/10^6 cells/μg/mL			
		−MA	(r^2)	+MA	(r^2)
Diesel	Cat	0.25	(0.96)	0.063	(0.78)
	Nissan	4.19	(0.88)	2.87	(0.86)
	Olds	1.21	(0.95)	1.28	(0.93)
	VW Rabbit	0.98	(0.89)	0.72	(0.64)
Gasoline, unleaded	Mustang	0.38	(0.98)	1.09	(0.81)
Gasoline, leaded	Ford Van	NEG[b]		5.60	(0.90)

[a]Assay performed with 6×10^6 cells in 10 mL for 4 hr.
[b]Highly toxic at less than 10 μg/mL.

All the diesel samples were mutagenic in the mouse lymphoma assay, and except for the Mercedes sample, all the diesel samples showed that the mutagenic activity was greater in the absence of the metabolic activation system. All the diesel organic samples were also more cytotoxic in the absence of metabolic activation than in its presence (*22*). The maximum increases in mutation frequency (two to four times the spontaneous frequency) occurred at concentrations ranging from 20 to 300 μg/mL. The gasoline catalyst Mustang sample was more mutagenic and cytotoxic in the presence of metabolic activation than in the absence of the activation system. Polycyclic aromatic hydrocarbons such as benzo(a)pyrene [B(a)P] are not mutagenic in this assay without the addition of the S9 metabolic activation system. Preliminary evaluation of 1-nitropyrene (95%) in this assay suggests that it also requires an exogenous metabolic activation system for activity.

BALB/c 3T3 Mutagenesis Assay

The BALB/c 3T3 mutagenesis assay was developed by Schechtman and Kouri (*24*) to measure simultaneously both mutagenic activity and morphological transformation. Forward mutation is measured by using ouabain resistance (*25*). Cells ($1–2 \times 10^6$) were exposed in suspension for 2 hr with increasing concentrations of the diesel organics dissolved in acetone. Curren et al. (*26*) assayed the Caterpillar, Nissan, and Oldsmobile diesel samples, and the Mustang gasoline sample in the BALB/c 3T3 mutagenesis assay. Although several individual doses of the diesel sample did induce a significant increase in ouabain-resistant mutants, none of the samples induced a dose-dependent increase in mutation frequency. A majority of the concentrations tested appeared to be above the limit of solubility as evidenced by insoluble material in the assay. This problem had not previously been encountered when DMSO was employed as a solvent with a similar sample. Curren et al. (*26*) assumed that all seven doses tested may have been similar due to the solubility limits. They combined all of the mutant colonies observed for a sample and divided it by the total number of surviving cells to determine a mutation frequency for the dose range tested. Using this method of analysis, both the Nissan sample and Mustang gasoline sample were highly mutagenic ($p < 0.05$) both without and with metabolic activation. The Oldsmobile diesel sample showed approximately a twofold increase in mutation frequency, which was not significantly different from the solvent control, and the Caterpillar diesel sample showed no increase in mutation frequency.

Chinese Hamster Ovary Mutagenesis Assay

The CHO assay measures forward mutation at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus by using 6-thioguanine resistance (*27*). The CHO assay was conducted with modifications by Casto et al. (*28*) to evaluate the mutagenic activity of the diesel and gasoline samples. Cells ($1.5–2 \times 10^6$) were treated with increasing concentrations of the organics dissolved in DMSO for 24 hours. These assays were only conducted in the absence of metabolic activation in two to three separate experiments. Reanalysis of the combined data with cell survivals above 10% using linear regression analysis showed a relatively weak to negative response for the Caterpillar, Oldsmobile, and Mustang samples. The samples that would be considered positive were the Nissan and VW Rabbit with activities of 0.16 ($r^2 = 0.73$) and 0.091 ($r^2 = 0.46$) mutation frequency/10^6 cells/μg/mL, respectively. Li and Royer (*29*) also have reported that the extractable organics from a series of diesel cars was generally very low in mutagenic activity in CHO cells with a slight increase in activity with metabolic activation. Simultaneous treatment of the CHO cells with a comutagen, e.g., B(a)P, caused

significant enhancement of the mutagenic activity (*29*).

Cheshier et al. (*30*) have shown that CHO cells readily phagocytize whole diesel particles, which become closely associated with the nucleus. Under these conditions, 100 μg/mL of diesel particles caused a tenfold increase in mutation frequency above the controls (*30*).

DNA Damage Assays

Saccharomyces cerevisiae D3 Recombinogenic Assay

The diploid yeast *S. cerevisiae* D3 can be used to measure mitotic recombination by scoring for red-pigmented mutant colonies formed in the presence of adenine (*31*). The mutants are generated from a recombinational event resulting from DNA breakage and repair after exposure to DNA-damaging chemicals. Initial studies on the diesel and comparative samples reported that no reproducible or dose-related responses were observed (*22*). Further studies of these samples in the *S. cerevisiae* assay showed that two of the three diesel samples assayed, the Nissan and VW Rabbit, did result in reproducible dose-related increases in mitotic recombinants. The Nissan sample caused 62 mitotic recombinants/10^5 surviving cells/μg/mL ($r^2 = 0.79$) without activation and 46 ($r^2 = 0.64$) with activation. The VW Rabbit caused 24 mitotic recombinants/10^5 surviving cells/μg/mL ($r^2 = 0.4$) without activation and 7.2 ($r^2 = 0.2$) with activation. The response was greater and more reproducible in the absence of metabolic activation. The Oldsmobile sample was weakly positive in the absence of metabolic activation, and the gasoline Mustang sample did not reproducibly increase the number of mitotic recombinants. Polycyclic aromatic hydrocarbons such as B(a)P do not induce mitotic recombination in *S. cerevisiae* (*22*), and NO_2-PNAs have not been examined in this assay.

DNA Strand Breaks in Syrian Hamster Embryo Cells

Damage to cellular DNA, which results in the formation of DNA fragments, can be measured directly by alkaline elution techniques (*32*). Casto has shown that chemical induction of DNA damage in primary Syrian hamster embryo (SHE) cells can be detected following centrifugation on alkaline sucrose gradients (*33*). The diesel Caterpillar, Nissan, Oldsmobile, and VW Rabbit samples were tested at four concentrations from 31 to 250 μg/mL. None of these samples produced a significant change in the sedimentation profile of DNA from the treated SHE cells. The gasoline Mustang sample did produce a significant increase in DNA strand breaks at the highest concentration tested (250 μg/mL). In comparing several *in vitro* tests to detect carcinogens in Syrian hamster cells, Casto suggests that the DNA strand breakage assay is the least sensitive of the assays evaluated (*28*).

Unscheduled DNA Repair in Liver Cells

The liver cell DNA repair assay measured autoradiographic unscheduled DNA synthesis in freshly isolated hepatocytes. The Oldsmobile diesel sample was evaluated in the hepatocyte primary culture/DNA (HPC/DNA) repair assay by Williams according to previously published procedures (*34*). Unscheduled DNA repair was induced from 10 to 100 μg/mL with an average of 36.7 grains/nucleus at 100 μg/mL. The response appeared to be dose-related; however, insufficient numbers of concentrations were tested in any one experiment to perform a regression analysis. Combination of the data from four separate experiments resulted in a slope of 0.325 grains/nucleus/μg/mL ($r^2 = 0.78$).

Sister Chromatid Exchange Assay in CHO Cells

The sister chromatid exchange (SCE) assay measured the increase in exchanges between two chromatids of each chromosome in cells grown in the presence of bromodeoxy-uridine (BrdU) during replication. The increase in SCEs observed after cells have been treated with chemical mutagens has been related to recombinational or postreplicative repair of DNA damage (*35*). The diesel and gasoline emission samples were tested in the SCE assay using the CHO cell system previously described (*22*). This method uses a 21.5-hr sample exposure period; however, due to the cytotoxic effects of the metabolic activation system, only a 2-hr exposure period was used when the samples were tested with metabolic activation. It is not possible, therefore, to compare directly the induction of SCEs with and without metabolic activation. The slope of the dose-response regression analysis is shown in Table 5.

All of the diesel and gasoline samples, except the Oldsmobile sample, induced SCEs in the absence of metabolic activation. In the presence of metabolic activation, all of the diesel samples induced SCEs except the Caterpillar sample. The significantly lower activity in the presence of activation is pre-

Table 5. Sister chromatid exchanges in CHO cells.

Fuel	Vehicle description	Slope: SCE/cell/μg/mL			
		−MA[a]	(r^2)	+MA[b]	(r^2)
Diesel	Cat	0.011	(0.83)	NEG	
	Nissan	0.30	(0.93)	0.071	(0.87)
	Olds	NEG		0.017	(0.46)
	VW Rabbit	0.075	(0.99)	0.030	(0.92)
Gasoline, unleaded	Mustang	0.076	(0.99)	NT[c]	

[a]−MA exposure 21.5 hr.
[b]+MA exposure 2 hr.
[c]NT = not tested.

Table 6. Summary of chromosomal aberrations in CHO cells.

μg/mL (Nissan)	12 hr after treatment		15 hr after treatment		21 hr after treatment	
	Total cells	Aberrations, %[a]	Total cells	Aberrations, %[a]	Total cells	Aberrations, %[a]
0	515	1.75	276	4.06	147	2.00
20	136	5.07	152	10.53	151	6.80
40	129	6.98	59	28.80	98	10.91
60	115	18.26	77	62.03	122	15.28
80	192	20.83	T[b]	T	88	22.72

[a]Percentage of cells with all types of aberrations.
[b]T = toxic.

sumably due in part to the much shorter exposure period. The polycyclic aromatic hydrocarbon [B(a)P] tested in this assay induced SCEs only when metabolic activation was added. Preliminary studies on 1-nitropyrene (95% pure) showed that it was weakly active in the absence of the metabolic activation system.

Chromosomal Aberrations

Chromosomal Aberrations in CHO Cells

Chromosomal aberrations that can be detected as a result of treatment of cells in culture include both numerical and structural aberrations. Scoring of numerical aberrations, however, is not generally recommended for this assay. Structural aberrations include breaks, deletions, gaps, exhanges, or translocations at chromosomal and/or chromatid levels. These aberrations are generally observed 6 to 24 hr after cell treatment. In order to determine the optimal time after treatment to observe aberrations, CHO cells treated with the Nissan sample for 6 hr were scored for structural chromosomal abnormalities at 12, 15, and 21 hr (*36*). A summary of those results is shown in Table 6. A dose-related positive response was observed at all three time periods.

Chromosomal Aberrations in Human Lymphocytes

Human lymphocytes freshly isolated from blood samples taken from normal individuals can be exposed to chemicals *in vitro* and analyzed for chromosomal aberrations. The diesel Oldsmobile sample was exposed to lymphocytes from two individuals at five doses ranging from 0.1 to 100 μg/mL with and without an S9 metabolic activation system. Chromosome aberrations were scored by McKenzie according to previously published criteria (*37*). In the absence of metabolic activation, treatment of lymphocytes with the diesel Oldsmobile sample resulted in a four- to fivefold increase in the percentage of cells with chromosomal aberrations over the dose range tested. Chromosome and chromatid breaks and aneuploidy were observed at 0.1 to 1.0 μg/mL. Chromosomal fragments, dicentrics, and endoreduplications were observed at doses above 5 μg/mL. Chromosomal and chromatid gaps were observed only at 100 μg/mL. In the presence of metabolic activation, no increase in the total percentage of cells with aberrations was observed, although an increase in chromosomal fragments and dicentrics was observed.

In Vivo Mutagenesis Bioassays

The organics extractable from motor vehicle particle emissions are mutagenic in many microbial and

mammalian cell assays, as described above. However, these assays are not readily applicable to testing whole emissions, nor can they test for the heritability of mutations. For these reasons, plants (Tradescantia), insect (Drosophila) and mammals (mice and hamsters) have been employed to evaluate the *in vivo* mutagenic activity of diesel emissions, as summarized in Table 7.

The Tradescantia micronucleus test and stamen hair gene mutation assays both have been shown to detect the mutagenic activity of volatile and gaseous chemicals and environmental emissions. Ma (*38*) reported that diluted diesel exhaust induced micronuclei (broken pieces of chromosomes) in Tradeantia. Whole diesel emissions were also shown by Schairer (*39*) to induce gene mutations in the Tradescantia stamen hair assay.

The fruit fly, *Drosophila melanogaster*, provides a well-defined genetic test system to measure inherited damage. Two independent investigatos (*40,41*) have evaluated the mutagenicity of whole diesel emissions by using the *D. melanogaster* sex-linked recessive lethal assay. Nix (*41*) also tested the gaseous emissions from filtered exhaust. Neither the whole nor filtered exhaust was found to induce mutations in this assay.

Whole animal rodent bioassays using mice or hamsters provide the opportunity to measure genetic damage (e.g., induction of micronuclei or induction of SCEs) in somatic cells as well as heritable genetic damage. Both mice and hamsters have been used in studies by Pereira (*42*) and Rounds (*43*) to measure induction of micronuclei and SCEs in bone marrow, lung cells and fetal liver after exposure to whole diesel emissions. In all of these studies except the lung cell SCE assay, the whole emissions were negative. After exposure to collected particles, the SCE assays were positive in both bone marrow and lung cells. All of these genetic damage assays in somatic cells were positive when the animals were treated with the organics extracted from diesel particles. These studies suggest that the organics associated with diesel particles are capable of inducing genetic damage in somatic cells in the lung, bone marrow, and fetal liver. However, under conditions where the animals were exposed to high concentrations of whole diesel exhaust for several months, only induction of SCEs in lung cells was observed. These results suggest that insufficient concentrations of the mutagenic organics would reach the germinal cells to cause heritable mutations.

Heritable mutations in mice after exposure to diesel exhaust were assayed for by Russell et al. (*44*) using the specific locus, dominant lethal and heritable translocation assays. The results in all the heritable mutagenesis assays were negative.

The *in vivo* mutagenesis studies further confirm the mutagenic activity of the organics associated with diesel particles, while showing the lack of transmitted genetic effects after animal exposure to whole diesel exhaust emissions. These findings suggest either that the mutagenic components do not reach the gonads or that the heritable genetic assays were insensitive to the frameshift mutagens present in diesel emissions. Polycyclic aromatic hydrocarbons and other frameshift mutagens such as the NO_2-PNAs have not been well studied in either the Drosophila or mouse heritable mutagenesis assays.

Carcinogenesis Bioassays

Oncogenic Transformation Assays

Chemically induced carcinogenesis is currently considered to be a multistep process that may involve

Table 7. *In vivo* mutagenicity of diesel emissions.

Bioassay system	End point	Reference	Whole emissions	Gases[a]	Particles[a]	Extractable organics[a]
Tradescantia	Micronucleus test	Ma et al. (*38*)	+			
Tradescantia mutation	Stamen hair gene assay	Schairer (*39*)	+	+		
Drosophila melanogaster	Sex-linked recessive lethal test	Schuler and Niemeir (*40*); Nix (*41*)	–	–		
Mouse	Micronucleus assay	Pereira (*42*)	–		–	+
Mouse	Bone marrow SCE assay	Pereira (*42*)	–		+	+
Mouse specific locus	Point mutation test	Russell et al. (*44*)	–			
Mouse dominant lethal	Chromosome damage test	Russell et al. (*44*)	–			
Mouse heritable translocation test	Chromosome damage test	Russell et al. (*44*)	–			
Chinese hamster	Micronucleus assay	Pereira (*42*)	–		–	(+)[b]
Syrian hamster	Lung cell SCE assay	Rounds (*43*)	+		+	+
Syrian hamster	Fetal liver SCE assay	Pereira (*42*)	–		–	+

[a]Where bioassays have not been conducted, no entry in the table is shown.
[b](+) = weakly positive.

DNA damage or mutation as an initial step. Oncogenic transformation assays measure the induction of morphological transformations that result in the formation of colonies of cells phenotypically similar to malignant cells (type III foci). These transformed cells generally cause tumors when injected into a syngeneic host.

Several of the diesel and gasoline samples in Table 1 have been tested in two oncogenic transformation assays. Curren et al. (*26*), using mouse embryo cells (BALB/c 3T3), found that all of the diesel and gasoline samples, except the Caterpillar sample, induced some transformed foci. Dose-related responses were not observed, which may be due to the problems discussed above with the BALB/c mutagenesis assay that was conducted simultaneously. Casto et al. (*28*), using primary SHE cells, found that none of the diesel or gasoline samples caused transformation in these experiments. Unfortunately, lack of induction of transformation by one of the positive controls and difficulties in obtaining acceptable lots of serum for these assays prevented further testing.

Viral Enhancement of Transformation

The viral enhancement assay measures the increased sensitivity of cells to virus-induced transformation. Although this assay is listed with the transformation assays, Casto et al. (*45*) have reported the significance of DNA damage and repair in the enhancement of viral transformation by chemicals. This assay may be a measure, therefore, of DNA damage. The viral enhancement of the transformation assay of Casto (*46*) was employed in the evaluation of the diesel, gasoline and several comparative samples (*29*). The transformation frequency was determined (number of transformed foci per 10^6 surviving cells) in at least three separate experiments. The dose-response curves for selected experiments were reported by Casto et al. (*28*). The combined data from all experiments were pooled and reanalyzed to determine the slope of the dose response. Concentrations resulting in less than 10% survival were not used in determining the slope of the transformation response, as shown in Table 8.

All of the diesel and gasoline samples, except the Caterpillar sample, increased the viral enhancement of transformation. The Oldsmobile and VW Rabbit samples were very weakly active, and the dose responses had low r^2 values, 0.68 and 0.25, respectively. The variation in response between the three separate experiments was significant, and even the Mustang gasoline sample, which caused a 0.33 transformation frequency/μg/mL, had an unacceptably low r^2 value of 0.18 for the pooled slope analysis. The Nissan sample caused a transformation frequency equivalent to the Mustang sample, with an r^2 of 0.76.

The variation in dose response observed for this assay may be due to the significant variation between different hamster embryo preparations in their response to carcinogens. For this reason each experiment was also analyzed separately and the experiment resulting in a slope with the highest r^2 is also reported in Table 8. The activity of the VW Rabbit sample is increased to 0.14 (r^2 = 0.98), and the activity of the Mustang is substantially decreased to 0.024 (r^2 = 0.88) by this analysis.

Skin Tumor Initiation

Mice treated topically with chemical carcinogens produce both benign (papillomas) and malignant (squamous cell carcinomas) tumors. The tumor-initiating activity of a chemical can be determined when mice are treated with a single application of the chemical and subsequently treated with a strong tumor promoter, i.e., 12-O-tetradecanoyl phorbol-13-acetate (TPA). Tumor-initiating chemicals are thought to induce somatic mutations as a result of covalent binding to DNA and other macromolecules (*47*). Nesnow et al. (*48–50*) have reported the detailed methods and results of skin tumor initiation studies on these diesel and gasoline extracts in SENCAR mice. The skin tumor-initiating activity

Table 8. Enhancement of viral transformation in Syrian hamster embryo cells.

Fuel	Vehicle description	Transformation frequency/μg/mL			
		Pooled[a]	(r^2)	Nonpooled[b]	(r^2)
Diesel	Cat	NEG		NEG	
	Nissan	0.328	(0.76)	0.42	(0.94)
	Olds	0.021	(0.68)	0.03	(0.91)
	VW Rabbit	0.059	(0.25)	0.14	(0.98)
Gasoline, unleaded	Mustang	0.33	(0.18)	0.024	(0.88)

[a]Slope determined from pooling the data from 2–3 separate experiments.
[b]Slope determined separately for individual experiments and the slope with the highest r^2 reported.

Table 9. Skin tumorigenesis in SENCAR mice.

Fuel	Vehicle description	Linear slope; papillomas/mouse/mg[a]	(r^2)	Model slope: papillomas/mouse/mg[b]	Papillomas/mouse at 1 mg[c]
Diesel	Cat	NEG		ND[d]	0.10
	Nissan	0.52	(0.99)	0.59	0.46
	Olds	0.14	(0.83)	ND	0.31
	VW Rabbit	0.30	(0.53)	0.19	0.24
Gasoline, unleaded	Mustang	0.085	(0.76)	0.17[e]	0.16

[a]Slope determined from linear regression analysis (*48*) and an average of males and females.
[b]Slope determined from nonlinear Poisson model with background correction (*49*) and an average of males and females.
[c]Values based directly on papilloma multiplicity data at 1 mg (*49*) and an average of males and females.
[d]ND = non-determinable.
[e]Data from males only (*50*).

to produce papillomas of these samples is shown in Table 9.

Papillomas were induced with all of the samples except the Caterpillar. Complete analysis of the tumor initiation activity and a discussion of the carcinogenic activity of these samples on mouse skin are reported by Nesnow et al. (*49*).

Summary and Discussion

Comparison of Mutagenic and Carcinogenic Activity of Extractable Organics from Motor Vehicle Particle Emissions in Various Bioassays

The organics extractable from motor vehicle particle emissions were found to be mutagenic in all three types of bioassays: gene mutation assays, DNA damage assays, and chromosomal aberration assays. The three mutagenesis assays that resulted in reproducible dose-response data and that also have been used to evaluate at least four organic emission samples are: *S. typhimurium* bacterial mutagenesis assay (Table 3), L5178Y mouse lymphoma mutagenesis assay (Table 4), and the SCE assay in CHO cells (Table 5). The relative activity of the diesel and gasoline organic emission samples has been compared between these three assays and the two short-term carcinogenesis assays, which resulted in reproducible dose-response data, enhancement of the viral transformation assay (Table 8) and mouse skin tumor initiation assay (Table 9).

In order to evaluate whether the relative activity of these samples correlated between assays, the activity determined from the slope of the dose-response for each sample in one assay was plotted versus that in the second assay. Linear regression analysis and confidence bands were determined as shown in Figure 1. The correlations, as indicated by the r^2 values (Table 10) for the gene mutation assays when plotted versus all the other assays, was very good (r^2>0.90) in the absence of metabolic activation, except for the mouse lymphoma and the skin tumorigenesis versus SCE in CHO cells. The addition of metabolic activation to the *S. typhimurium* assay decreased its correlation with both viral enhancement and skin tumorigenesis. The viral enhancement assay, which is thought to be dependent upon DNA breakage to allow increased frequency of virus insertion, correlated highly (r^2>0.96) with the mutagenesis assays in the absence of metabolic activation. The mouse lymphoma assay both with and without metabolic activation correlated highly (r^2 = 0.95) with the skin tumorigenesis assay.

These studies suggest that there is generally good agreement both qualitatively and quantitatively between the short-term mutagenesis and carcinogenesis bioassays in which a dose-related response is observed for the organics extractable from diesel

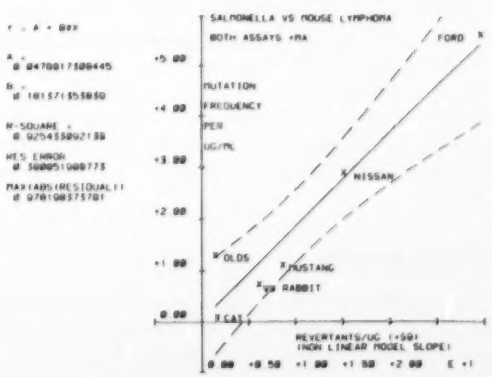

FIGURE 1. Linear regression analysis of the mutagenic activity of diesel and gasoline samples in the *S. typhimurium* mutagenesis assay versus the L5178Y mouse lymphoma assay. Confidence bands are shown in broken lines.

Table 10. Correlation of dose-response slopes of short-term bioassays for diesel and gasoline emission samples.

Bioassay comparison	Exogenous metabolic activation	r^2
Salmonella vs. mouse lymphoma	-MA	0.96
Salmonella vs. mouse lymphoma	+MA	0.93
Salmonella vs. SCE in CHO	-MA	0.98
Salmonella vs. SCE in CHO	+MA	0.94
Salmonella vs. viral enhancement	-MA	0.99
Salmonella vs. viral enhancement	+MA	0.79
Salmonella vs. skin tumorigenesis	-MA	0.90
Salmonella vs. skin tumorigenesis	+MA	0.72
Mouse lymphoma vs. SCE in CHO	-MA	0.84
Mouse lymphoma vs. SCE in CHO	+MA	0.87
Mouse lymphoma vs. viral enhancement	-MA	0.96
Mouse lymphoma vs. viral enhancement	+MA	0.83
Mouse lymphoma vs. skin tumorigenesis	-MA	0.95
Mouse lymphoma vs. skin tumorigenesis	+MA	0.95
SCE in CHO vs. viral enhancement	-MA	0.96
SCE in CHO vs. viral enhancement	+MA	0.93
SCE in CHO vs. skin tumorigenesis	-MA	0.83
SCE in CHO vs. skin tumorigensis	+MA	0.83
Viral enhancement vs. skin tumorigenesis	-MA	0.92

[a]Exogenous metabolic activation (S9) added to one or both assays is shown as +MA; when no exogenous metabolic activation system was added to either assay, it is shown as -MA.

and gasoline emission particles. Several assays (e.g., DNA strand breaks and oncogenic transformation in SHE cells) do not detect activity in these samples. Other assays (e.g., mutagenesis and oncogenic transformation in BALB/c 3T3 cells) did provide qualitative data to indicate that these organics were active; however, reproducible dose-related responses were not observed. This result may be due to a lack of increasing amounts of chemical reaching the cell as the exposure concentration increased, probably as a result of solubility problems with these complex mixture samples. More solubility problems arose in those *in vitro* assays where acetone rather than DMSO was used as a solvent.

Conclusions

The studies reviewed here were undertaken to evaluate the mutagenicity of organics associated with motor vehicle particle emissions in a battery of mammalian cell bioassays. These data provide strong evidence that these organics are mutagenic in mammalian cells. Furthermore, the relative activity of a series of emission extract samples, which exhibit approximately one order of magnitude range inactivity in the *S. typhimurium* bacterial mutagenesis assay, exhibits a similar range in activity in mammalian cell assays. These studies suggest bacterial mutagenesis assays are useful in providing a relative ranking of mutagenic activity from different motor vehicle emissions which highly correlates with the relative activity in mammalian cell mutagenesis and skin tumor initiation assays.

Since a significant portion of the bacterial mutagenic activity in diesel emissions appears to be due to NO_2-PNA compounds, particularly mono- and dinitrated pyrenes, more studies are needed to evaluate the activity of these compounds in mammalian cells. Preliminary studies on the activity of nitropyrenes suggest that these compounds are active in mammalian cells. The concentrations of mono- and dinitrated pyrenes (*51*) in the samples tested here, however, can not account for all of the "direct-acting" mutagenic activity observed in mammalian cells treated with diesel particle organics. Further research is needed to identify other mutagenic and potentially carcinogenic constituents of diesel and gasoline emissions.

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